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Involvement of the Endocannabinoid System in the Development and Treatment of Breast Cancer

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14. ABSTRACT
Work looking at the interaction of the synthetic cannabinoid WIN55,212-2 and ionizing radiation has led to preliminary results implicating a novel site of action in the MCF-7 breast cancer model. WIN55,212-2 has been shown previously to dose dependently, and potentially more importantly stereoselectively, inhibit the growth of breast cancer cells. Interestingly, when selective cannabinoid receptor antagonists AM251 and AM630 were administered, we see a failure to antagonize WIN55,212-2's antiproliferative effects. This despite the observation the MCF-7 cells express mRNA for the cannabinoid receptor CB2, which WIN55,212-2 has been shown to act on in other breast cancer cell lines. Further studies were conducted that pharmacologically excluded the involvement of members of the PPAR receptor system, known to be reactive to WIN55,212-2. TRPV1 is reported to be sensitive to some cannabinoids as well, and subsequently was evaluated and then excluded based on similar pharmacological experiments. Studies from a colleague have implicated the involvement of sphingosine-1-phosphate receptors as a potential site of action for WIN55,212-2 in the brain. Our work has since shown WIN55,212-2 to be able to antagonize this sphingosine-1-phosphate receptor system, and it's known importance to MCF-7 cell growth gives a potential mechanism of action. This interaction has not previously been reported and suggests a novel site of action that could be exploited with future research.

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Introduction

I am currently a predoctoral candidate at Virginia Commonwealth University working toward a Ph.D. in the Department of Pharmacology and Toxicology under the guidance of mentors Dr. David Gewirtz (primary) and Dr. Aron Lichtman (secondary). This grant is supporting my current research on a project, initiated through the Department of Defense Breast Cancer Research Program, to evaluate the utility of cannabinoids as treatments against breast cancer. A closely related goal is to determine whether the use of cannabinoids might interfere with the effectiveness of breast cancer therapies. The primary training I am receiving in addition to an in-depth understanding of current and proposed treatment of breast cancer includes the proper use of the scientific method for experimental design and technical execution at the bench. In addition, the training also heavily focuses on the communication aspects of science involving literature review, oral communication, written communication and formal presentation either through poster or slideshow based talks.

Body

During the course of the past year, the efforts on this project were dedicated to evaluating the interaction of cannabinoid compounds with radiation in breast cancer. The cannabinoid-based medications Marinol (Δ9-tetrahydrocannabinol; THC) and Cesamet (Nabilone) are clinically approved by the USA Food and Drug Administration for the suppression of nausea and vomiting associated with chemotherapy and radiation treatment; however, the potential interaction of these compounds with either radiation or chemotherapy has not been reported to our knowledge. Using preclinical in vitro models of breast cancer cell growth, we found that the various cannabinoids do not interfere with the anti-proliferative effects of either treatment; furthermore, the synthetic cannabinoid, WIN55,212-2 (WIN2), augmented the antiproliferative effects of radiation in three breast cancer cell lines.

WIN2, derived from the aminoalkylindole series, is one of the most highly studied synthetic cannabinoids. It has been shown to produce the full spectrum of psychoactive effects associated with marijuana use (Compton et al. 1992). WIN2 is an agonist at both cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) (Howlett et al. 2002). In vitro studies assessing GTPγS activity have shown that WIN2 acts as a full agonist at CB1 (Sim-Selley et al. 2002). Interestingly, both CB1 and CB2 have been implicated in the antiproliferative effects of various cannabinoids in different model systems. In glioma cancer cells, THC has been shown to inhibit cell growth by activating CB1 (Salazar et al. 2009), while WIN2 has been shown to inhibit breast cancer cell growth by activation of CB2 (Qamri et al. 2009). However, as indicated below, our studies raise serious reservations relating to the transferability of these findings to MCF-7 breast cancer cells.

RT- PCR was used to confirm the expression of message for the CB1 and CB2 receptors. Figure 1A shows clear expression of CB2 and an extremely faint signal for CB1 mRNA. In order to determine if WIN2 inhibits breast tumor growth through these cannabinoid receptors, studies were performed using the respective highly selective competitive CB1 and CB2 antagonists, AM251 and AM630. However, neither antagonist attenuated WIN2's antiproliferative effects (Figure 1B).

In view of these findings indicating that CB1 and CB2 are not responsible for WIN2's effects, further studies were designed to address other possible targets that might mediate WIN2's antiproliferative effects. Reports have shown that cannabinoids including WIN2 are capable of activating members of the peroxisome proliferator-activated receptor (PPAR) family (O'Sullivan 2007). Mostly these receptors are associated with lipid

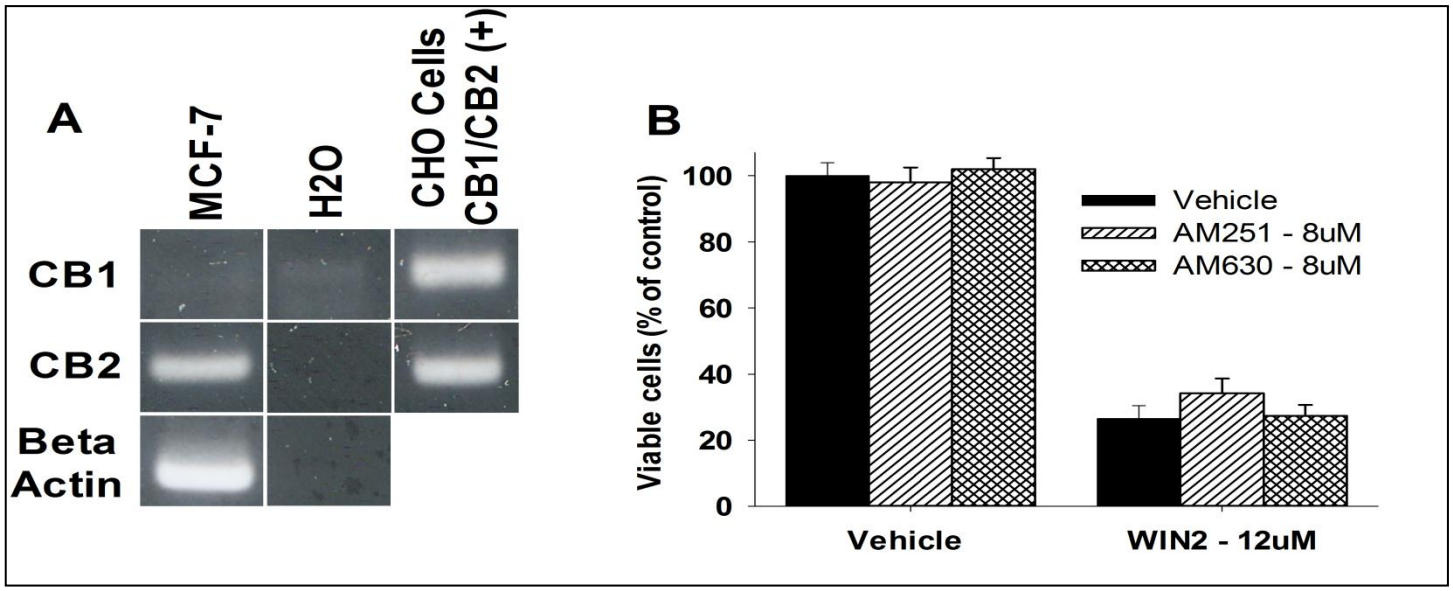

Figure 1 - WIN2's antiproliferative actions are not mediated by cannabinoid receptors CB1 or CB2. RT-PCR performed on MCF-7 cells using primers for CB1, CB2 and Beta actin. H_2O was used as a negative control and CHO cells overexpressing human CB1 or CB2 were used as a positive control. Representative images shown **(A).** Cannabinoid receptor antagonists selective for CB1 (AM251) and CB2 (AM630) were given at 8μM concurrently with either vehicle control or 12μM WIN2. MCF-7 cells were analyzed at 96hrs for cell viability using trypan blue exclusion **(B).** Quantitative data presented as mean±se with an n=3. WIN2 significantly reduced cell proliferation; however, the antagonists did not produce significant effects when given alone and did not affect the anti-proliferative effects of WIN2.

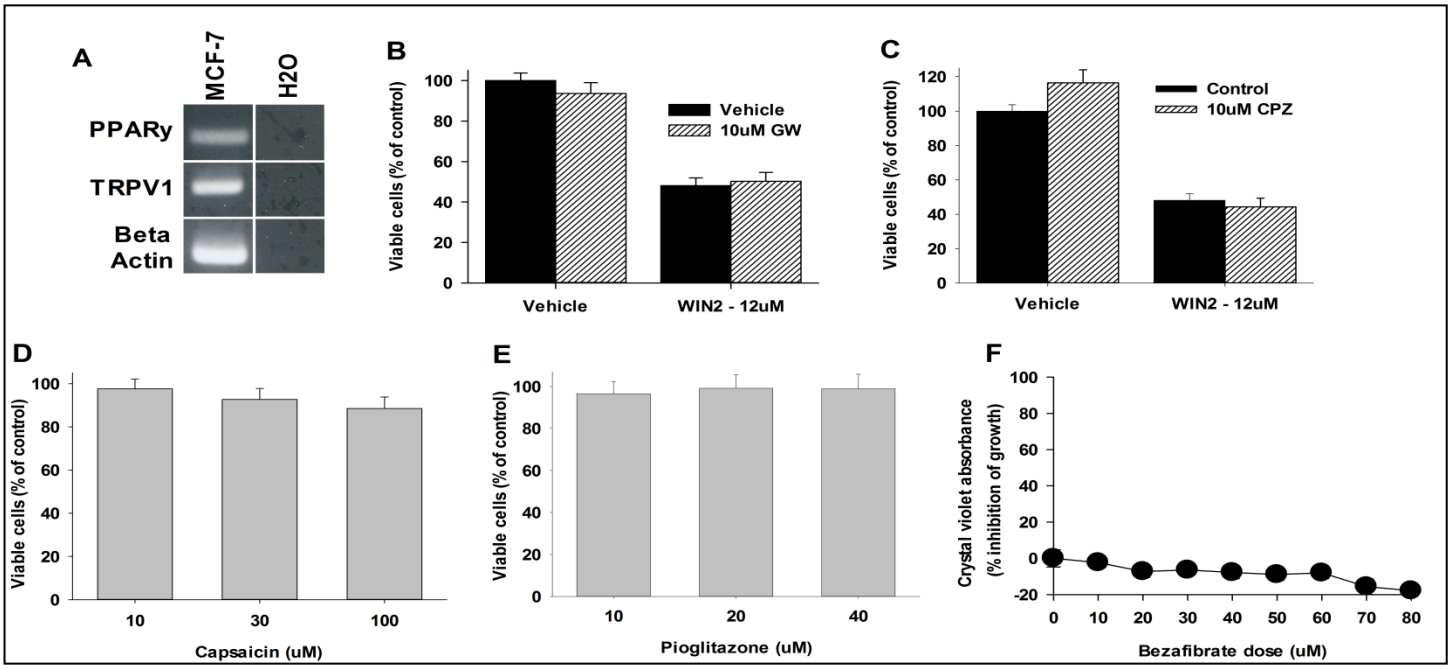

Figure 2 - WIN2's antiproliferative actions are not mediated by the vanilloid channel TRPV1 or members of the PPAR family. Experiments performed in MCF-7 cells. **(A)** RT-PCR performed using primers for TRPV1, PPARγ and Beta actin. H_2O was used as a negative control. Representative image shown. **(B)** PPARγ and **(C)** TRPV1 antagonists, GW9662 (10μM) and Capsazepine (10μM) respectively, were given in combination with either vehicle or 12μM WIN2. Dose responses were administered of **(D)** the TRPV1 agonist Capsaicin (10, 30 and 100μM) and **(E)** the PPARγ agonist Pioglitazone (10, 20 and 40μM). **(F)** Additionally, cells were treated with a dose response of the pan-PPAR agonist Bezafibrate with doses between 0 and 80uM. **(B,C,D and E)** Cell viability was assessed using trypan blue exclusion. **(F)** Proliferation was assessed using the crystal violet assay. Quantitative data presented as mean±se with an n=3. No relevant significant differences were detected.

metabolism and drugs acting at these receptor sites are currently used in the treatment of diabetes (O'Sullivan 2007). Additionally, we considered the cation channel vanilloid receptor 1 (TRPV1) as a potential drug receptor. Other cannabinoids, specifically anandamide, have been shown to act at this site (Pertwee et al. 2010).

Figure 2A shows that RT-PCR confirmed the expression of message for PPARγ and TRPV1. Figure 2B/C indicates that the antagonist capsazapine (TRPV1) and GW-9662 (PPARγ) did not inhibit WIN2's antiproliferative effects. Both potential receptor targets were further evaluated using TRPV1 and PPARγ agonists. We hypothesized that if activation of these receptors mediated WIN2's antiproliferative actions, then agonists for these receptors should also be able to inhibit MCF-7 cell growth. However, as shown in Figures 2 D/E, neither the TRPV1 agonist Capsaicin nor the PPARγ agonist pioglitazone inhibited MCF-7 cell growth. The pan-PPAR agonist Bezafibrate also failed to inhibit the growth of MCF-7 cells (Figure 2F). Taken together with our previous experiments that demonstrated unequivocal stereospecificity of WIN2 in inhibiting breast tumor growth in three different tumor cell lines, these studies indicate that WIN2 might be acting through a unique and previously unidentified receptor pathway.

Recent unpublished work from a collaborator's laboratory has suggested the possibility that WIN2 might be acting through the sphingosine-1-phosphate (S1P) receptor. In brains from CB1 KO mice, WIN2-stimulated GTPγS binding was antagonized by S1P receptor antagonists, while other cannabinoids tested were ineffective in CB1 knockout brains. When this GTPγS stimulation was compared to S1P, a full agonist at S1P receptors, it was seen that WIN2 was less efficacious by comparison suggesting actions as a partial agonist (data unpublished). Parallel work from the lab of Dr. Sarah Spiegel's and others has shown that S1P receptors are intimately involved in various process in cancer cells including proliferation, cell fate decisions and migration (Olivera et al. 1999, Spiegel et al. 2003, Pyne et al. 2010). As a partial agonist at S1P receptors, WIN2 would also be capable of acting as partial antagonist within this system.

Our preliminary results suggest the possible involvement of S1P receptors in WIN2's anti-proliferative actions in MCF-7 cells. Experiments have shown that overexpression of sphingosine kinase, the rate limiting enzyme for S1P synthesis can stimulate growth in various cell lines (Olivera et al 1999). We show here that under low serum conditions, exogenous administration of S1P stimulates growth of MCF-7 cells, and when WIN2 is co-administered with the S1P, the growth stimulating effects are antagonized (Figure 3A). The studies presented in Figure 3B confirm these findings where MCF-7 cell growth stimulated by the synthetic S1P

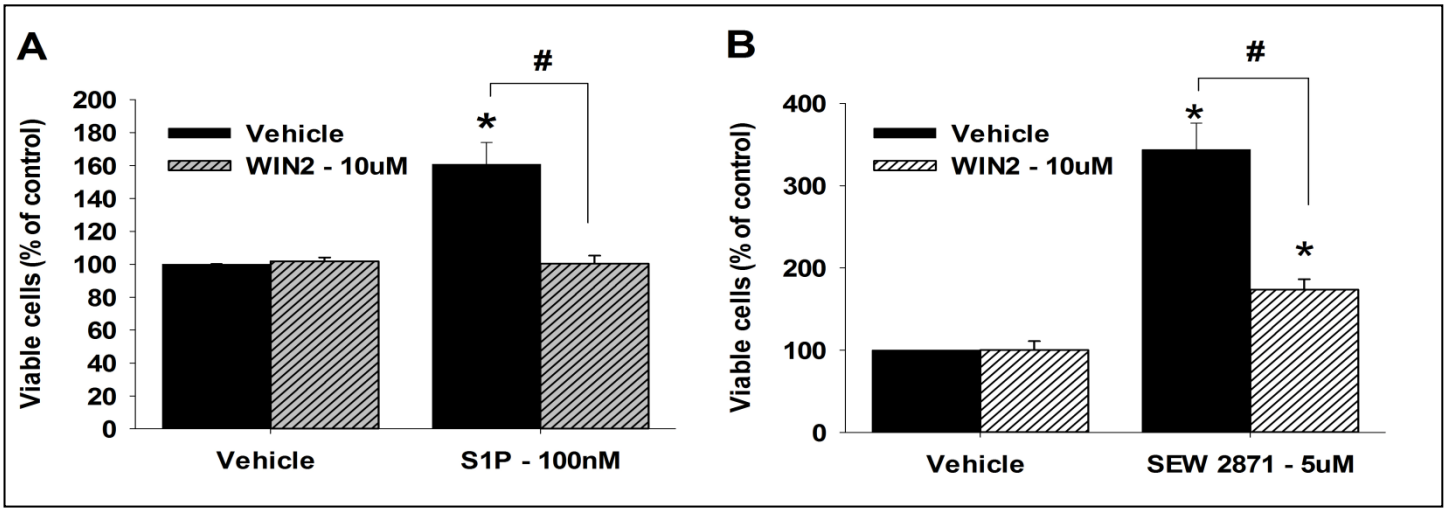

Figure 3 - WIN2 antagonizes the growth stimulating effects of S1P receptor agonists. MCF-7 cells were treated with either **(A)** vehicle or 100nM sphingosine-1-phosphate or **(B)** vehicle or 5uM SEW2871. Concurrently cells were treated with either vehicle or 10uM WIN2. Cell viability was assessed using trypan blue exclusion. Data converted to % of control and presented as mean±se with an n=3 (* = $p<0.05$ vs veh-veh and # = $p<0.05$).

receptor agonist SEW2871 is suppressed by WIN2. As this experiment was performed under serum-replete conditions, a higher dose of WIN2 was required to suppress growth stimulation.

In summary, we have found that WIN2 augments the antiproliferative effects of radiation in various breast cancer cell lines. Additionally, our preliminary evidence suggests that these effects might be mediated through S1P receptors. These results are exciting on multiple levels. The discovery of a novel site/mechanism of action opens possibilities for the development of novel treatments that could potentially offer increased efficacy, increased potency, or both. Furthermore, WIN2 possesses antinociceptive actions in preclinical models of cancer pain (Guerrero et al. 2008) and can suppress radiation induced-emesis in the least shrew (Darmani et al. 2007). All of these characteristics together suggest that WIN2 or drugs with similar properties could prove to be valuable adjuvant therapy to radiation. Moreover, other cannabinoids, including THC and nabilone, do not interfere with the anti-proliferative effects of radiation on MCF-7 breast cancer cells.

Key research accomplishments

WIN55,212-2's evaluation at cannabinoid receptors

- RT-PCR has confirmed the presence of CB2 mRNA in MCF-7 cells
- Selective cannabinoid receptor antagonists AM251 (CB1) and AM630 (CB2) failed to inhibit the antiproliferative effects of WIN55,212-2

WIN55,212-2's evaluation at PPAR and TRPV1 receptors

- RT-PCR confirmed the presence of PPARy and TRPV1 mRNA in MCF-7 cells
- The PPARy selective antagonist GW-9662 failed to antagonize WIN55,212-2
- The TRPV1 antagonist Capsazepine failed to antagonize WIN55,212-2
- The PPARy agonist pioglitazone and the TRPV1 agonist capsaicin were unable to recapitulate the growth inhibitory effects of WIN55,212-2
- The pan-PPAR agonist bezafibrate was unable to recapitulate the growth inhibitory effects of WIN55,212-2

WIN55,212-2's evaluation at S-1-P receptors

- WIN55,212-2, at sub-lethal doses, was able to antagonize the growth stimulatory effects of the sphingosine-1-phosphate receptor agonists sphingosine-1-phosphate and SEW2871

Reportable outcomes

Abstracts submitted to

- Carolina Cannabinoid Collaborative Conference
- Virginia Academy of Science
- American Association of Cancer Research
- Pharmacology and Toxicology Research Retreat

Presentations

- Carolina Cannabinoid Collaborative Conference – Presentation – “Enhanced Antiproliferative Actions of Combined Radiation and WIN55,212-2 on MCF-7 Breast Cancer Cells: Exploration of a Mechanism of Action” – Greenville, North Carolina
- Virginia Academy of Science – Presentation – “The Interaction Between WIN55,212-2 and Radiation on Inhibiting the Growth of Breast Cancer Cells” – Norfolk State University, Norfolk, Virginia
- American Association of Cancer Research – Poster – “The Cannabinoid WIN55, 212-2 Enhances the Response of Breast Cancer Cells to Radiation” – Chicago, Illinois
- Pharmacology and Toxicology Research Retreat – Poster – “Combining Cannabinoids and Radiation in Breast Cancer” – Williamsburg, Virginia

Conclusion

Experiments presented here have pushed us to the tentative conclusion that the WIN55,212-2 is inhibiting the growth of MCF-7 cells through novel actions at the S-1-P receptor system. This observation provides many exciting possibilities that most obviously start with the development of new drug therapies. S-1-P system research is still in its infancy but has suggested promising advances in cancer therapy, and this discovery could unveil a novel drug structure capable of aiding the fight. Additionally, WIN55,212-2 also retains it well characterized actions at both CB1 and CB2 receptors. Depending on the cancer model evaluated either CB1 or CB2 has been implicated in antagonizing cancer growth preclinically. WIN55,212-2's actions at two receptors, S-1-P and cannabinoid, could suggest greater efficacy than drugs targeted at either individual site of action. Finally, WIN55,212-2's actions at CB1 receptors also offer palliative treatment possibilities. Agonists capable of activating CB1 receptors have been linked to alleviation of cancer bone pain, as well as, chemotherapy and radiation associated emesis in preclinical models. This could offer a third facet to WIN55,212-2's mechanism of action, which might not only improve patient survival but also quality of life. Extensive research is still needed before any of these possibilities can be realized in the form of a novel therapeutic.

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